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Intraepithelial, lamina propria and Peyer's patch lymphocytes of the rat small intestine: isolation and characterization in terms of immunoglobulin markers and receptors for monoclonal antibodies

NICOLE LYSCOM & M. J. BRUETON *Department of Child Health, Westminster Medical School, London*

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Summary. Methods have been determined for the isolation, purification and subsequent characterization of separate populations of rat intestinal lymphoid cells, namely intraepithelial (IEL), lamina propria (LPL) and Peyer's patch lymphocytes (PPL). Dissociation of the epithelium from the basement membrane with subsequent release of IEL was achieved by citrate buffer incubation followed by vortex agitation. LPL were released from the remaining tissue by scraping, and PPL were similarly obtained. Some preparations of lamina propria were further subjected to collagenase digestion. After filtration and density gradient centrifugation, average yields of 220×10^4 IEL, 54×10^4 LPL and 220×10^4 PPL per gram of gut were obtained. Immunofluorescence characterization demonstrated that cells bearing the MRC OX8 (T-suppressor) marker predominated in IEl (73%) and were present in lower concentrations in LPL (26%) and PPL (6%). Cells with the W3/25 (T-helper) marker accounted for a small proportion of each of the lymphocyte preparations. IEl were unusual in containing a population of cells which were negative for the W3/13 marker for T cells, but were MRC OX8 positive. B lymphocytes were present in PPL (55%) and LPL (31%), but were virtually absent in IEL (less than 1%). Few plasma cells were observed. The techniques described will allow functional investigations to be made and lead to a better understanding of mucosal immunity.

Abbreviations: IEL, intraepithelial lymphocytes; LPL, lamina propria lymphocytes; PPL, Peyer's patch lymphocytes; T_H, T helper; Ts, T suppressor; CB, citrate buffer; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DTT, dithiothreitol; SIg, surface immunoglobulin; LPLc, LPL prepared with collagenase; Ig, immunoglobulin.

Correspondence: Ms N. Lyscom, Department of Child Health, Westminster Children's Hospital, Vincent Square, London SW1.

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INTRODUCTION

It has long been recognized that normal immune responses to dietary antigen include antibody secretion into the gut lumen (Besredka, 1919) and specific systemic tolerance (Chase, 1946; Swarbrick, Stokes & Soothill, 1979). More recently dysfunction of these defence mechanisms has been studied in relation to the incidence of autoimmune disease (Ammann & Hong, 1971), allergic disorders (Soothill, Stokes, Turner, Norman & Taylor, 1976) and gastrointestinal infections (Ross & Asquith, 1979).

Peyer's patch lymphocytes (PPL) are particularly responsive to gut antigen. They give rise to specific antibody-producing cells in the lamina propria (Craig & Cebra, 1971) and to systemic suppressor cells

(Mattingley & Waksman, 1978; Ngan & Kind, 1978; Elson, Heck & Strober, 1979). The role of intraepithelial lymphocytes (IEL) is poorly understood and the lymphocyte subpopulations represented amongst them are also uncertain. Their number is diminished in young animals (Ferguson & Parrott, 1972) and in malnourished individuals (Maffei, Rodrigues, De Camargo & Campara, 1980), both being situations in which mucosal immunity is disturbed. Contrary to this, elevated IEL numbers are observed in jejunal enteropathies associated with coeliac disease and in some cases of cows' milk protein intolerance and giardiasis (Mavromichaelis, Brueton, McNeish & Anderson, 1976). Whether these increases reflect the cause or the effect of these disorders is not clear. Some investigations have been carried out on stained sections of gut tissue (Selby, Janossy & Jewell, 1981b; Selby, Janossy, Goldstein & Jewell, 1981a), but further characterization of these cells, particularly from a functional point of view, has been hampered by the difficulties involved in the isolation and purification of gut lymphocytes, and in particular in the separation of the IEl population from the lamina propria.

The purpose of this investigation was to develop the technique initiated by Brueton (1977) for the separation and purification of rat IEL and to determine a satisfactory method for the preparation of rat lamina propria lymphocytes (LPL). The cells prepared by these methods, together with PPL have been characterized by immunofluorescence for surface and cytoplasmic immunoglobulins and for receptors for the W3/13, W/25 and MRC OX8 monoclonal antibodies, which are considered to be specific for T cells, T-helper (T_H) and T-suppressor (Ts) cells, respectively (Brideau, Carter, McMaster, Mason & Williams, 1980). The techniques developed should allow functional investigations to be made and lead to a better understanding of mucosal immune function.

MATERIALS AND METHODS

Animals

Category 3 PVG rats (PVG/Ola) of 130–230 g (8–15 weeks old) were obtained from Olac Ltd, Bicester.

Media

The citrate buffer (CB) used contained 50 mM trisodium citrate, 96 mM sodium chloride, 8 mM potassium dihydrogen orthophosphate, 5·6 mM di-sodium hydrogen orthophosphate and 1·5 mM potassium chloride. The phosphate-buffered saline (PBS) contained 145 mM sodium chloride, 7·2 mM di-sodium hydrogen orthophosphate and 1 mM sodium dihydrogen orthophosphate with 3 mM sodium azide. Both media were of pH 7·2 and were supplemented with penicillin (100 i.u./ml), streptomycin (100 μg/ml) (Glaxo Laboratories Ltd) and bovine serum albumin (BSA; fraction V, Armour Pharmaceutical Co. Ltd) at 0·25% (w/v).

Isolation of mucosal lymphocytes

This procedure is outlined in Fig. 1. Rats were anaesthetized with ether and killed by cervical dislocation. The small intestine was immediately removed, flushed with CB and slit open. The Peyer's patches were carefully dissected out and the gut was divided into segments. Both the Peyer's patches and the segments were incubated in dithiothreitol (DTT, 1 mM, Sigma Chemical Co., Ltd) in CB for 3 min at room temperature to remove mucus, after which they were thoroughly rinsed in CB. Cells were released from the Peyer's patches by scraping with a scalpel. Incubation of the gut segments in CB/BSA for 15 min at 37° with gentle agitation followed by vortex agitation for 30 seconds separated the basement membrane from the epithelium. The gut segments were removed, rinsed in fresh medium and scraped with a glass slide to release lamina propria cells.

When collagenase digestion was applied, segments of gut or mucosal scrapings obtained after the removal of the epithelium were incubated under sterile conditions in RPMI 1640/BSA containing 20 u./ml of collagenase (type IV, Sigma Chemical Co. Ltd). Incubation was carried out in a 37° water bath with continuous stirring for various periods of time.

Purification of mucosal lymphocytes

IEL, LPL and PPL in CB/BSA at 4° were filtered through cotton gauze to remove coarse clumps and subsequently passed through cotton wool filters. The latter consisted of multiple Pasteur pipettes packed loosely with cotton wool (about 5 mm in length) above the constriction and cut off below it. All filters and glassware were rinsed carefully with cold medium at each stage in order to minimize the loss of adherent subpopulations.

The cells were further purified by density gradient centrifugation using Lymphoprep (density 1·077, Nyegaard and Co. A/S, Oslo) at 500 *g* for 30 min at 18°. The cells recovered at the interface were washed

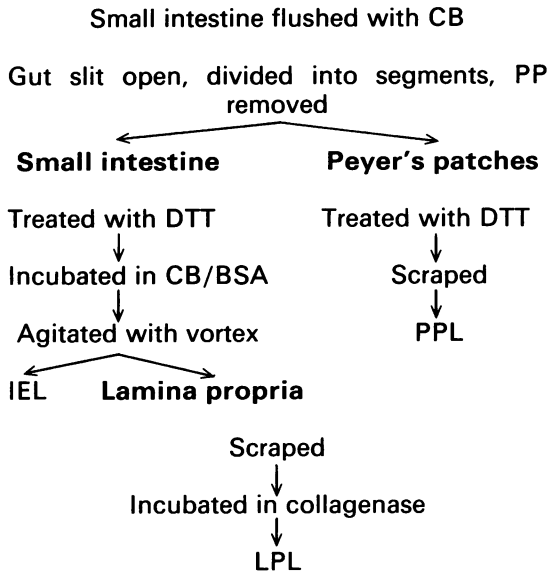

Figure 1. Isolation of mucosal lymphocytes.

three times at 200 *g* at 4°, once for 20 min and twice for 10 min, and then resuspended in PBS/BSA for investigation. An alternative medium of density 1·09 studied for gradient centrifugation consisted of twelve parts of 14% (w/v) Ficoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and five parts of 32·8% (w/v) sodium metrizoate (Isopaque, Nyegaard and Co.). Both constituents were dissolved in distilled water.

Cell recovery and viability was determined by trypan blue exclusion (0·05% in saline) using a haemocytometer counting chamber.

Preparation of cells from thymus and spleen

Cells were released from these tissues by teasing with a scalpel and forceps in PBS/BSA. They were filtered through cotton gauze and washed twice before use.

Immunofluorescence staining

B cells with surface immunoglobulins (SIg) were labelled in a direct assay using fluorescein-conjugated anti-rat immunoglobulin (Wellcome Reagents Ltd, London). They were incubated at 4° for 20 min and washed twice.

Cells bearing T-cell markers were labelled in an indirect assay using the mouse monoclonal antibodies W3/13, W3/25 and MRC OX8 (Seralab Ltd. Crawley Down, Sussex). For these assays, crossreacting antigens were masked by prior incubation of the cells with a rabbit anti-rat IgG serum (Wellcome Reagents Ltd). After one wash the cells were incubated with either one or a combination of the monoclonal antibodies at saturating dilutions. This was followed by four further washes. Cells binding these antibodies were subsequently labelled with fluorescein-conjugated anti-mouse IgG (Miles Labs. Ltd, Slough) and washed twice. All incubations were for 20 min and all procedures were carried out at 4°. Control samples were treated similarly but without the addition of a monoclonal antibody. The preparations were suspended in mounting medium consisting of nine parts glycerol and one part PBS for microscopic investigation under ultra-violet light.

The proportion of plasma cells in the samples was determined using cytocentrifuge preparations. The slides were fixed in methanol at 4° for 15 min, rinsed in PBS and labelled with fluorescein-conjugated anti-rat immunoglobulin (Wellcome Reagents Ltd) for 45 min in humid conditions at room temperature. The slides were rinsed four times over a period of at least 2 hr before the addition of mounting medium for examination.

Cytochemical staining

Gut segments were embedded in paraffin wax for

sectioning and stained with Harris' haematoxylin and 1% eosin. Giemsa/May-Grünwald staining for fixed cytocentrifuge preparations was carried out as described by Hudson & Hay (1976). Cells were stained for non-specific esterase using the technique developed by Yam, Li & Crosby (1970).

RESULTS

Preparation of IEL and LPL

The average yield of IEL obtained after incubation and vortex agitiation for 30 sec was 940×10^4 viable lymphocytes per gram of gut. Longer periods of agitation caused damage to the basement membrane and subsequent release of LPL. This was determined by histological examination. Sections of gut obtained before the isolation procedure and after the removal of the epithelium are shown in Fig. 2.

Preliminary investigations had demonstrated that treatment of gut segments with the mucolytic agent DTT increased the yield of IEL and reduced contamination with mucus and unwanted cells. The chosen temperature and time of incubation of segments in CB/BSA was optimal with respect to IEL yield and purity. The presence of BSA which was used at this and all subsequent stages was found to enhance cell viability.

The mechanical method used to release LPL gave a yield of 180×10^4 viable LPL per gram of gut. Removal of most of the lamina propria was confirmed histologically. Incubation of mucosal scrapings in

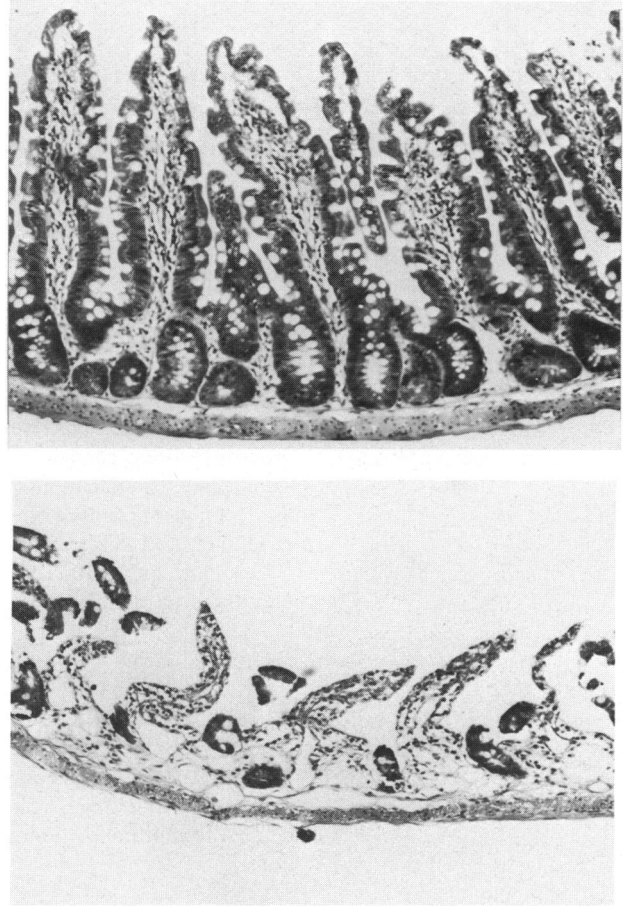

Figure 2. (a) Untreated rat small intestine. (Magnification $\times 40$.) (b) Rat small intestine after the removal of the epithelium. (Magnification $\times 40$.)

Table 1. Cellular contaminants before and after the purification procedure and mucosal lymphocyte recovery ($n = 5$)

	IEL		LPL		LPLc	
	before	after	before	after	before	after
Contaminants/100 viable lymphocytes						
Dead cells	230	29	1203	195	2398	73
Enterocytes	43	9	20	5	64	52
Clumps	37	3	154	41	419	28
Lymphocytes $\times 10^4$/gram of gut	940	220	180	54	242	61

collagenase for 15 min but no longer, increased the yield of lymphocytes (LPLc, Table 1). However, it appeared that the additional procedure rendered the LPL more labile as this increase was not apparent at the end of the purification procedure. Incubation in collagenase of unscraped segments obtained after the removal of the epithelium (Bland, Richens, Britton & Lloyd, 1979; Davies & Parrott, 1980) resulted in complete loss of cell viability long before the enzymic digestion was complete.

Purification procedure

The initial IEL and LPL preparations contained unacceptable numbers of contaminating dead cells, enterocytes and clumps. The extent of the removal of the unwanted cellular contaminants and lymphocyte recovery from the purification procedure is outlined in Table 1. IEL and LPL recoveries averaged 23% and 30%, respectively, representing average yields of 220×10^4 viable IEL and 54×10^4 viable LPL per gram of small intestine. The few clumps remaining at the end were small, each containing less than three cells.

Some lymphocytes were lost during cotton gauze and cotton wool filtration (30% IEL and 11% LPL on average). The most serious lymphocyte loss occurred during density gradient centrifugation (52% IEL and 48% LPL). A denser gradient (density 1·09) as described by Parish & Hayward (1974) did not improve recovery and the removal of contaminants was less efficient. Similar cell losses were encountered in the preparation of LPLc. Unpurified LPLc were much more contaminated than preparations obtained without collagenase. However, the former separated out better during density gradient centrifugation with a clearer ring formation at the interface and an improved elimination of dead cells and clumps.

After purification PPL yields averaged 220×10^4 viable lymphocytes per gram of gut. Contaminatior was minimal.

Immunofluorescence characterization of lymphocytes

The percentage of lymphocytes from the epithelium, lamina propria, Peyer's patches, spleen and thymus which were labelled by anti-rat antibodies are shown in Table 2. MRC OX8 positive cells (Ts) predominated in IEL (73%) and were present in lower concentrations in LPLc (32%) and PPL (6·4%). W3/25 positive cells (T_H) were present in all three mucosal lymphocyte preparations, accounting for 13% of IEL, 16% of LPLc and 10% of PPL. The results from experiments in which the MRC OX8 and W3/25 markers were used separately and together indicated that there was no significant overlap between cells binding these markers in the mucosal lymphocyte preparations. The W3/13 antibody which is considered to be a T-cell marker labelled only 29% of IEL. This is significantly lower than the value of 79% obtained for IEL using MRC OX8 and W3/25 antibodies in combination ($P = < 0{\cdot}001$). Results for IEL using W3/13, W3/25 and MRC OX8 antibodies in combination were higher than those obtained using W3/25 with MRC OX8 only, which suggests that the W3/13 antibody was taken up by some cells which were not labelled by the W3/25 and MRC OX8 antibodies.

B cells were virtually absent in IEL (0·6%) but were more abundant in LPLc (28%) and PPL (55%). Few plasma cells were observed in IEL (0·6%) in PPL (0·3%) or in LPLc (1·6%). Studies with anti-rat immunoglobulin antibodies and W3/13 antibodies used separately and together indicated that mucosal cells bearing these markers did not overlap.

Paired t tests between simultaneous assays demonstrated that lymphocytes obtained with and without collagenase showed a similar distribution of

Table 2. Percentages of lymphocytes (±1 standard deviation) from normal rat tissues labelled by specific antibody markers

Source of cells	*n*	Antibody								
		Anti-rat Ig (surface)	Anti-rat Ig (cytoplasmic)	W3/13	Anti-rat Ig (surface) + W3/13	MRC OX8	W3/25	MRC OX8 + W3/25	MRC OX8 + W3/25 + W3/13	Control (anti-mouse IgG)
IEL	5	0·6±0·9	0·6±0·8	29·2±6·0	32·0±13·1	73·0±4·7	12·6±2·6	79·0±5·0	85·2±4·3	0
LPL	8	30·5±13·3	2·6±1·8	33·3±15·1	68·9±9·7	26·0±14·2	15·0±8·5	42·5±14·5	52·0±13·3	0
LPLc	6	27·6±12·9	1·6±1·5	30·7±13·4	57·5±7·6	32·3±13·7	16·3±11·0	46·7±17·4	49·3±17·0	0
PPL	5	55·0±9·5	0·25±0·5	18·2±4·7	73·2±8·2	6·4±4·4	10·0±3·2	19·2±3·9	18·8±2·6	0·2±0·5
Spleen	10	50·1±5·1	1·3±0·6	45·7±7·9	92·4±4·2	23·3±7·2	28·4±10·9	47·6±5·4	52·3±8·8	0·3±0·5
Thymus	7	0·3±0·5	0·1±0·2	96·9±2·3	98·9±0·9	79·3±8·0	80·2±8·0	97·0±2·0	99·0±1·2	0

Table 3. Percentages of lymphocytes (±1 standard deviation) from normal rat spleen labelled by anti-rat antibodies in cell preparations obtained with and without the purification procedure

Method used	Antibody					
	Anti-rat Ig (surface)	Anti-rat Ig (cytoplasmic)	W3/13	MRC OX8	W3/25	Control (anti-mouse IgG)
Control procedure	52·0±5·8	0·5±0·3	44·4±9·0	25·8±5·0	32·2±4·8	0
Procedure for mucosal lymphocytes	58·4±7·4	0·8±0·3	42·8±10·6	29·0±5·9	28·8±6·7	0

lymphocyte subpopulations. The conditions used for these tests gave results for splenic and thymic lymphocytes which are in general agreement with those of Brideau *et al.* (1980).

Cytochemical staining

Granular lymphocytes as determined by Giemsa/-May-Grünwald staining of lymphocyte preparations were found to account for 31% of IEL and 19% of LPLc. These cells were virtually absent in PPL and spleen preparations. Very few plasma cells, polymorphonucleocytes and macrophages were observed amongst mucosal cells. Eosinophils accounted for 5·6% of IEL and 6·5% of LPLc, but they were virtually absent from Peyer's patches. The use of collagenase did not effect the distribution of cells observed.

The effect of the isolation and purification procedure on cell subpopulations

Comparison between spleen cell populations obtained by simple dissociation and filtration, with populations 'processed' by the purification procedure used to prepare mucosal lymphocytes showed no significant differences in the distribution of cells obtained with respect to the uptake of the markers applied (Table 3). Esterase staining confirmed that there was no difference in the proportion of macrophages obtained.

DISCUSSION

The observation that the majority of IEL exhibited surface markers for the MRC OX8 antibody is of particular interest in view of the latter's association with suppressor activity (Brideau *et al.*, 1980), but it was surprising that most of these cells did not take up the W3/13 T-cell marker used. It was noticeable that B cells were virtually absent amongst IEL while cells with the W3/25 antibody were present in small numbers. Nevertheless, cells with receptors for W3/13, W3/25 and MRC OX8 and for anti-surface immunoglobulin were present in LPL and PPL, although few cells with cytoplasmic immunoglobulin were present in any of these mucosal lymphocyte preparations.

Evidence for a true separation of IEL from LPL and PPL was provided by the complete absence of B cells from the former, a population which accounted for approximately 29% of LPL and 55% of PPL, respectively. The absence of granulated cells from the PPL confirmed that they were not contaminated by other mucosal lymphocytes.

The poor yield of LPL in relation to IEL was disappointing considering the predominance of LPL seen in stained histological sections. Incubation of mucosal scrapings in collagenase only marginally improved the initial LPL yield. Cell death was probably responsible for much of the loss encountered during purification. Rat mucosal lymphocytes were noted to be exceedingly labile, possibly because of the presence of enzymes released from the rapidly disintegrating enterocytes. For this reason it was not possible to obtain viable lymphocytes by means of collagenase digestion of unscraped mucosal segments as described for human tissue (Bland *et al.*, 1979).

Bland *et al.* (1979) pointed out that isolation techniques may be selective for different subpopulations, however, collagenase did not alter the distribution of cells labelled by the markers used in this study. Selective depletion of subpopulations, particularly B lymphocytes and macrophages which are adherent, was not observed when the purification procedure was applied to spleen cells.

The literature to date suggests that many IEL are

T-dependent. This was demonstrated by the paucity of IEL in thymus-deprived animals (Fichtelius, Yunis & Good, 1968) and supported by antibody labelling techniques (Guy-Grand, Griscelli & Vassalli, 1978; Selby *et al.*, 1981b) and functional assays (Arnaud-Battandier, Bundy, O'Neill, Bienenstock & Nelson, 1978). In this investigation most of the IEL exhibited Ts markers, a finding which is substantiated by recent studies on human tissue sections by Selby and his colleagues (1981b). The virtual absence of B lymphocytes in IEL is in agreement with Guy-Grand *et al.* (1978) and Selby *et al.* (1981a) but contradicts others (Chiba, Bartnik, Remine, Thayer & Shorter, 1981). In this investigation the IEL contained a large proportion of cells (68%) which were labelled by neither the B- nor by the T-cell marker. Many of these SIg and W3/13 negative cells were labelled by the MRC OX8 antibody, indicating that they were likely to be of T-cell origin. Using different markers for T lymphocytes, similar non-T, non-B cells have been observed in the mouse (Parrott, 1976) and in the human (Chiba *et al.*, 1981). Parrott (1976) suggested that these negative cells are in fact T lymphocytes and Guy Grand *et al.* (1978) attributed them to a population of T cells in the process of differentiation into mast cells.

Recent investigations by Mayrhofer (1980) demonstrated the presence of granulated lymphoid-like cells in the gut of thymus-deficient animals. He observed a close correlation between these cells and mucosal mast cells, and suggested that they were non-lymphoid precursors of mucosal mast cells of bone marrow origin. Granulated cells accounted for 31% of IEL in this investigation and results from experiments in which all three monoclonal antibodies were used in combination suggest that at least some of these granulated cells bear receptors for these markers.

Plasma cells with cytoplasmic immunoglobulin accounted for approximately 2% of LPL. A similar value was obtained in the rabbit by Rudzik & Bienenstock (1974) but in human gut higher values have been reported both in tissue sections (Bartnik, Remine, Chiba, Thayer & Shorter, 1980) and in cell suspensions (Goodacre, Davidson, Singal & Bienenstock, 1979). Few plasma cells were identified amongst LPL on morphological grounds by Eade, St. Andre-Ukena, Moulton, MacPherson & Beeken (1980). Bartnik *et al.* (1980) found few plasma cells amongst the LPL released from human colon. It was striking that the percentage of B cells in their cell suspensions was comparable with the number of plasma cells identified in tissue sections.

Functional assays have determined that LPL can effect proliferative, cytotoxic and suppressor cell-mediated immune responses (Singal, O'Neill, Clancy & Bienenstock, 1976; Davies & Parrott, 1980; Goodacre & Bienenstock, 1981), as well as antibody production to gut antigens (Robertson & Cooper, 1972). The presence of the cell type involved is confirmed by the surface marker studies described.

The distribution of cellular subpopulations observed amongst PPL correlates well with their understood function of antigen recognition and development into plasma cells in the lamina propria (Parrott, 1976), and association with specific systemic suppressor cells (Mattingley & Waksman, 1978; Ngan & Kind, 1978; Elson *et al.*, 1979).

The presence of IEL with T-helper markers and the close proximity of IEL to macrophage pseudopodia observed by Collan (1972) suggests that these cells may be involved in the initiation of cellular and humoral immune response to gut antigen. Cytotoxic and suppressor functions are attributed to splenic MRC OX8 positive lymphocytes which are also W3/13 positive (Cantrell, Robins, Brooks & Baldwin, 1981; Brideau *et al.*, 1980). Such cells were observed amongst IEL in this study and Arhaud-Battandier *et al.* (1978) showed that IEL are indeed capable of natural killer activity.

An important question arising from this work is the classification of these IEL which are MRC OX8 positive but W3/13 negative. It is conceivable that they are T cells in an unusual stage of differentiation, or alternatively they may represent cells of non-lymphoid origin. Their functional capacity as suppressor cells is unknown. Further characterization of gut lymphocytes from individuals susceptible to defects in mucosal immunity, together with functional studies should lead to a better understanding of mucosal defence mechanisms and their limitations.

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